



Research article

The preparation and antioxidant activities of three phenyl-acylchitooligosaccharides

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ABSTRACT

Chitooligosaccharides with two different molecular weights are acylated with three containing benzene carboxylic acids: salicylic acid (BHA), α -naphthylacetic acid (NAA) and indole-3-butyric acid (IBA) to obtain α -hydroxybenzoyl-chitooligosaccharide, α -naphthylacetyl-chitooligosaccharide, and 3-Indolebutyryl-chitooligosaccharide. The structure of the derivatives was characterized by FT-IR spectroscopy, ^{13}C NMR spectroscopy and elemental analysis. According to several amide characteristic absorption peaks between 1750 cm^{-1} – 1500 cm^{-1} in the FT-IR spectrum, it can be determined that the target group has been successfully grafted. And there are obvious characteristic absorption peaks of aromatic ring at 900 – 650 cm^{-1} . The six chemical shifts of 98.02, 76.42, 74.83, 72.00, 60.39, 55.37 ppm in ^{13}C NMR proved that the chitooligosaccharide did not destroy its own sugar ring structure during the reaction. The antioxidant activities of the derivatives, such as hydroxyl radical ($\cdot\text{OH}$) scavenging ability, superoxide anion ($\text{O}_2^{\cdot-}$) scavenging ability, reducing ability, and DPPH radical scavenging ability were investigated using various established systems. Comparing with chitooligosaccharide and containing benzene carboxylic acids, most derivatives have strong scavenging ability toward superoxide anions and DPPH radicals, and the clearance rate up to 47.44% and 80.27% separately. The reducing ability and hydroxyl free radical scavenging ability of the derivatives are only 0.032 Abs and 11.43%. The above results showed that the antioxidant activity of some derivatives was higher than that of chitooligosaccharide. The water solubility of the new derivatives was also greatly improved than that of containing benzene carboxylic acids. Therefore, the application of phenyl-acyl-chitooligosaccharide in antioxidants has laid a foundation, and has certain potential application value in the fields of medicine and agriculture and animal husbandry.

1. Introduction

According to several studies, oxidative stress in living organisms can lead to aging and chronic diseases [1]. Free radicals are molecules with one or more unpaired electrons. The unpaired electrons are unstable and have high reactivity. Under induced conditions such as drugs, inflammation, stress and drinking, unpaired electrons are released and free radicals accumulate in the body, which can easily cause oxidative damage to the body, damage cell membranes, proteins and nucleic acids, disrupt the normal physiological functions of the body, and lead to a series of chronic diseases [2, 3]. The degree of oxidative damage in the body can be regulated by antioxidants. Antioxidants refer to any type of substances that can scavenge free radicals and delay or prevent oxidation reactions, including synthetic antioxidants and natural antioxidants [4].

With the more extensive and in-depth research in the field of antioxidants, the advantages of natural antioxidant polysaccharides have been highlighted [5, 6, 7, 8].

Chitooligosaccharides are oligomers formed by glucosamine, N-acetylglucosamine or a combination of the two linked by β -1,4 glycosidic bonds [9, 10, 11, 12]. Chitooligosaccharides have the advantages of small molecular weight, good water solubility, non-toxicity, and good biocompatibility [13]. It has been widely used in chemical industry, medicine, food, agriculture, etc. [14]. Chitooligosaccharide is abundant in nature and is a natural antioxidant polysaccharide.

Salicylic acid (BHA) is an important raw material in the pharmaceutical industry and an intermediate in the preparation of aspirin. Studies have shown that salicylic acid exerts anti-inflammatory and broad-spectrum antibacterial effects in the field of dermatology [15]. α -Naphthylacetic

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acid (NAA) is commonly used in plant growth regulation and can speed up plant wound healing. Because α -naphthalene acetic acid can promote cell division and expansion. Xu et al. conducted in-depth research on the effect of α -naphthalene acetic acid on the cuttings of *Impatiens lingzhiensis*, and found that the rooting rate and survival rate of *Impatiens lingzhiensis* were significantly improved [16]. 3-Indolebutyric acid (IBA) is also a commonly used plant growth regulator that promotes cell division. In the study of Chen et al., 3-indolebutyric acid significantly improved the quality of litchi pressed roots, and the rooting speed was very fast. The above three containing benzene carboxylic acids have good biological activity, but the water solubility is poor. In this paper, three new types of phenyl-acylchitoooligosaccharides were prepared for the purpose of linking through new groups. Not only can new biological activities be generated, but also the antioxidant activity of chitoooligosaccharide can be improved, and the hydrophobicity of containing benzene carboxylic acids can be reduced. Experiments evaluated their antioxidant activity, and the results showed that the antioxidant activity of some derivatives was higher than that of chitoooligosaccharide, and the water solubility was greatly improved. Therefore, the new derivatives have certain potential application value in the fields of medicine and agriculture and forestry.

2. Chemistry

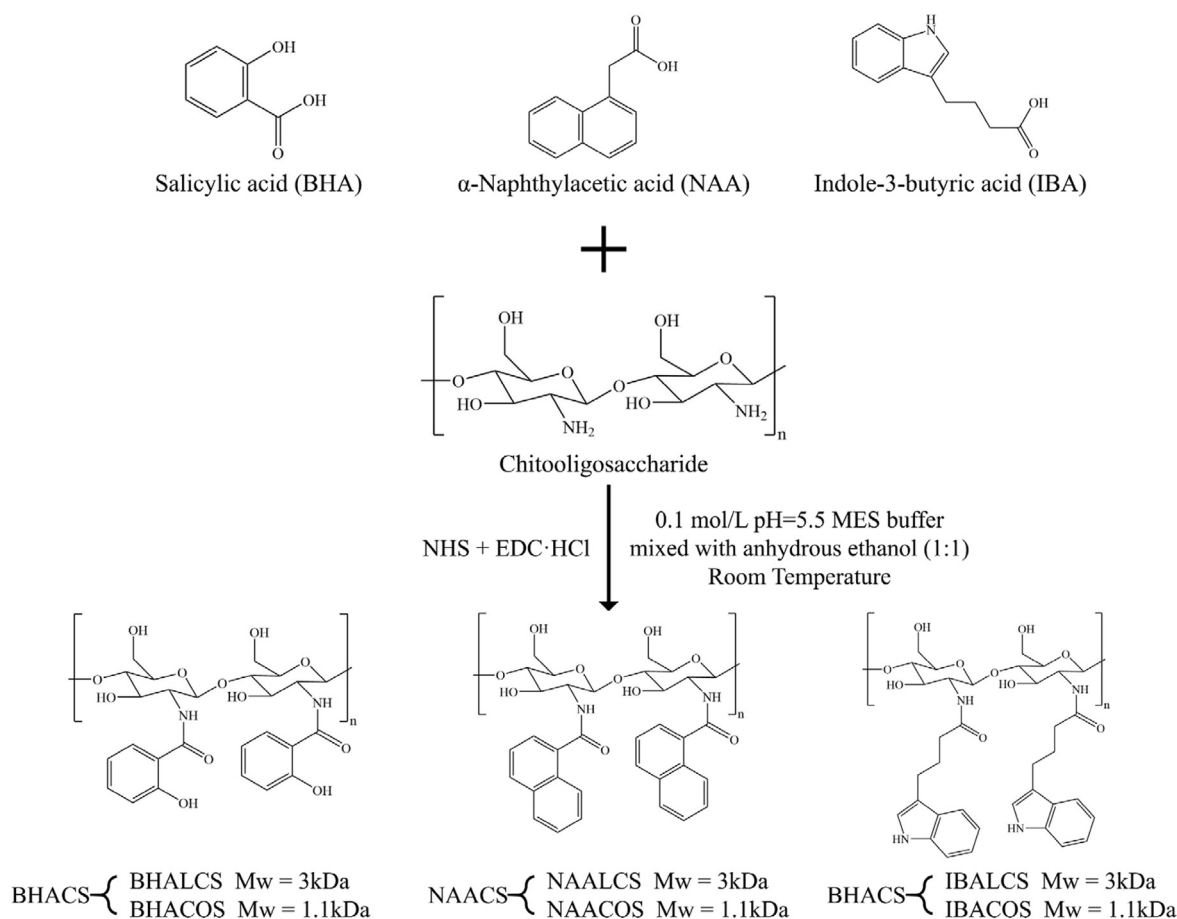
Phenyl-acylchitoooligosaccharide were synthesized as shown in Scheme 1. Chitoooligosaccharides with two different molecular weights were mixed with three containing benzene carboxylic acids [salicylic acid (BHA), α -naphthylacetic acid (NAA), indole-3-butylacetic acid (IBA)] in pH = 5.5 2-morpholineethanesulfonic acid (MES) buffer solution was

mixed with anhydrous ethanol at room temperature the reaction produces derivatives. During the reaction, the role of 1-ethyl-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC-HCl) and N-hydroxysuccinimide (NHS) is to let the carboxyl group Condenses with primary amines. All products gave satisfactory spectral data, fully conforming to their assigned structure. The antioxidant activity of the compounds was evaluated in an in vitro solution system.

3. Experimental

3.1. Materials

Low molecular weight chitosan (LCS) is a commercial material provided by Qingdao Yunzhou Biotechnology Co., Ltd. (China). It's deacetylation degree >90%, average molecular weight (Mw) of 3 kDa. Chitoooligosaccharide (COS) with a molecular weight of 1.1 kDa was prepared in our laboratory by the combined degradation of microwave and acetic acid. Tris (Tris), Reduced Coenzyme I (NADH), Safranin O, Nitrotriazolium Blue (NBT), Phenazine Potassium Sulfate (PMS), 1,1-Diphenyl-2-Trinitrophenylhydrazine (DPPH) was purchased from Sigma Chemicals Co. Disodium hydrogen phosphate dodecahydrate, sodium dihydrogen phosphate dihydrate, ferrous sulfate, disodium ethylenediaminetetraacetate (EDTA), absolute ethanol, 30% hydrogen peroxide (H_2O_2), 1-ethyl-(3-Dimethylaminopropyl)carbodiimide hydrochloride (EDC-HCl), N-hydroxysuccinimide (NHS), 2-morpholineethanesulfonic acid (MES), salicylic acid (BHA), α -naphthylacetic acid (NAA), indole-3-butylacetic acid (IBA). All other chemicals and reagents were of analytical grade and were used without further purification.



Scheme 1. Synthesis pathway of phenyl-acylchitoooligosaccharide.

3.2. Analytical methods

The infrared spectra of COS and its derivatives were detected using a Spectrum 65 (PerkinElmer) Fourier-Transform Infrared (FTIR) Spectrometer (Inner Mongolia Agricultural University, Hohhot, China) ranging from 4000 to 400 cm^{-1} at 25 °C. The ^{13}C nuclear magnetic

resonance (NMR) spectra were recorded at room temperature using a "MAS"-Magic Angle Spinning model test (Qingdao Xinfei Testing Co., Ltd., Qingdao, China). Elemental analyses were performed on a Leeman EA3000 elemental analyzer Inner Mongolia Agricultural University. All the samples were weighed using a Mettler XP6 millionth balance. The sample weights ranged from 1.5 to 2.5 mg. The degrees of substitution

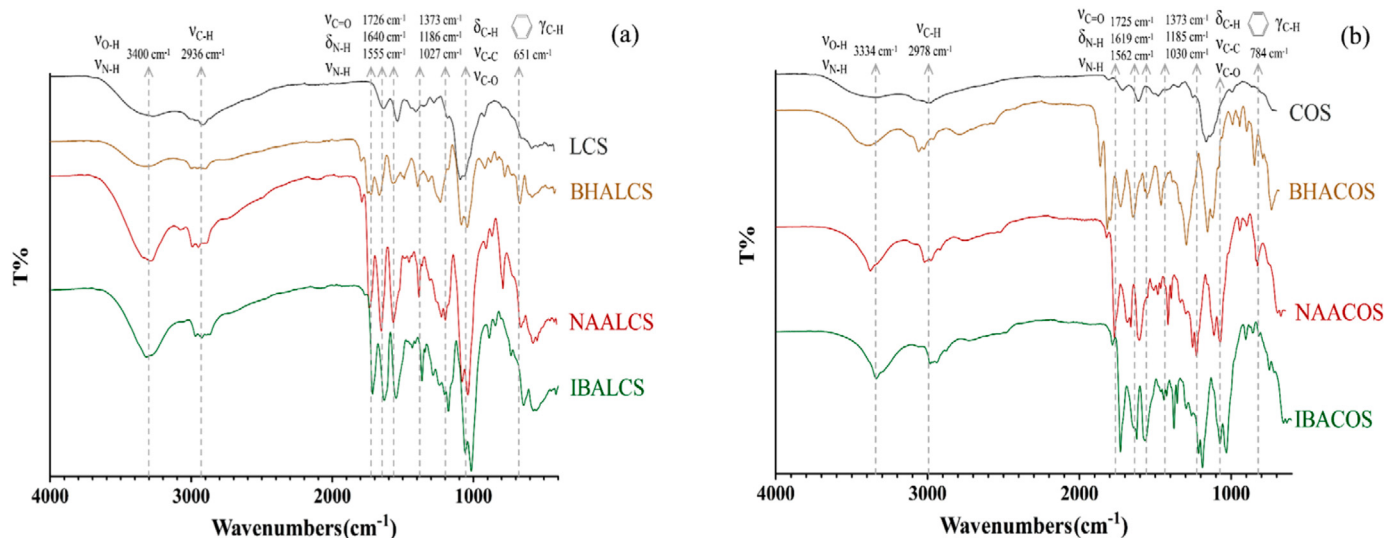


Figure 1. FT-IR spectrum data of LCS, PALCS, COS and PACOS. [(a) FT-IR spectrum data of LCS and PALCS, (b) FT-IR spectrum data of COS, PACOS].

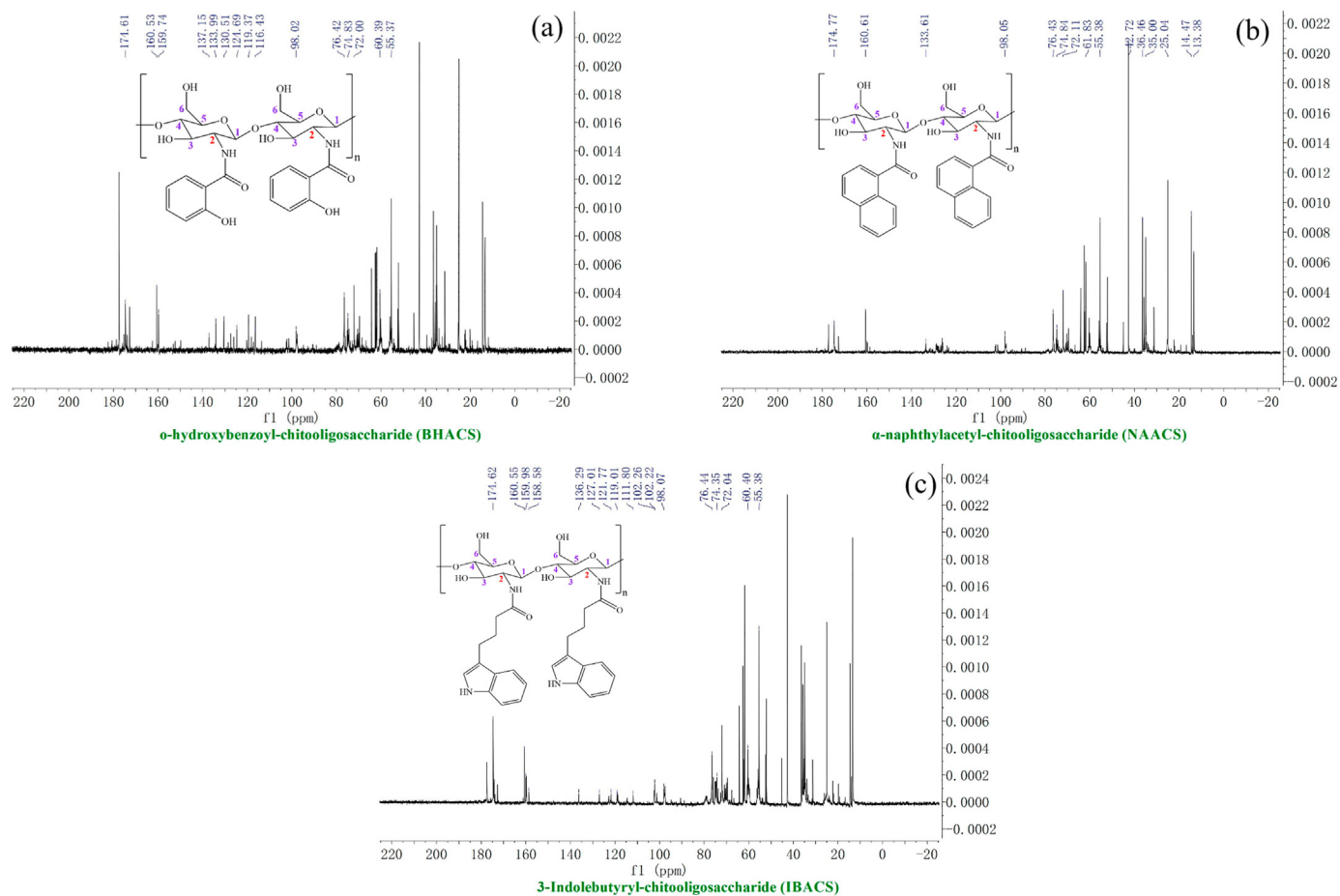


Figure 2. ^{13}C NMR spectrum data of BHACS, NAACS and IBACS. [(a) ^{13}C NMR spectrum data of BHACS, (b) ^{13}C NMR spectrum data of NAACS, (c) ^{13}C NMR spectrum data of IBACS].

Table 1. Elemental analysis results, yield and the grafted degree of chitoooligosaccharide derivatives.

Compounds	Yield (%)	Elemental analysis (%)						DS (%)
		Theoretical value			Experimental value			
		C	H	N	C	H	N	
CS	-	44.67	6.95	8.68	41.76	8.70	8.56	-
BHACS	46.84	55.32	5.69	4.96	51.12	10.33	6.15	74.47
NAACS	41.11	65.65	5.80	4.26	52.19	10.97	11.83	28.62
IBACS	49.18	58.96	9.85	8.09	52.42	10.38	10.11	71.12

(DS) were calculated in accordance with the carbon to nitrogen (C/N) ratios of COS and its derivatives. The DS of chitoooligosaccharide derivatives were estimated employing the following formula [17, 18]:

$$DS = \frac{m(C/N)_1 - (C/N)_0}{n}$$
, where $(C/N)_1$ is the C/N of the modified COS, $(C/N)_0$ is the C/N of the original COS, and m and n are the numbers of nitrogen and carbon atoms, respectively, introduced by the COS modification.

3.3. Preparation of phenyl-acylchitoooligosaccharide

The molar ratio of containing benzene carboxylic acid and chitoooligosaccharide was set as 20:1 and 7:1 with reference to the polymerization degree of chitoooligosaccharide. The containing benzene carboxylic acid was dissolved in MES- ethanol buffer solution (pH = 5.5) with stirring until complete dissolution. EDC·HCl and NHS were added to

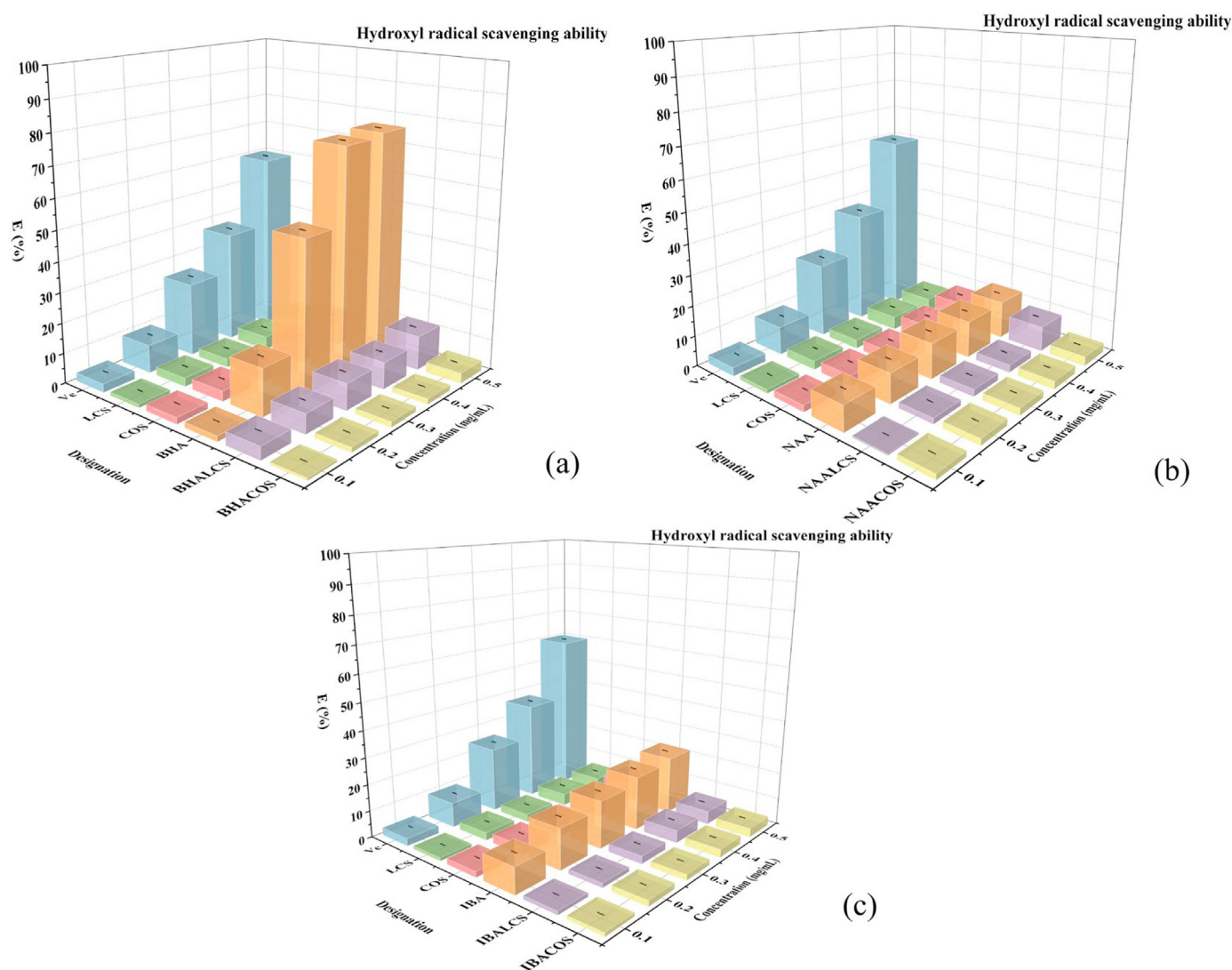


Figure 3. Scavenging effects of BHACS, NAACS and IBACS on hydroxyl radical. [(a) scavenging effects of BHACS, (b) scavenging effects of NAACS, (c) scavenging effects of IBACS].

continue stirring for 3 h to activate the carboxyl group (the molar ratio of containing benzene carboxylic acid: EDC·HCl: NHS = 1:3:3). Then, chitooligosaccharide was added and stirred at room temperature for 24 h. The solution was dialyzed against distilled water for 48 h using a 500 Da MW cut-off dialysis membrane. Then rotate the solution in the dialysis bag to 30–50 mL (the concentration temperature is controlled below 55 °C). Finally, the concentrated solution was freeze-dried for more than 48 h to obtain a tawny or brown target product powder.

3.4. Hydroxyl radical assay

The hydroxyl radicals scavenging ability of COS and phenyl-acylchitooligosaccharide were assessed by the method of Zhong et al. [19]. The scavenging ability was calculated as follows:

$E\% = \left(\frac{A_{sample} - A_{blank}}{A_{control}} \right) \times 100$; among them, the A_{sample} is absorbance value of the experimental sample; the $A_{control}$ is absorbance value of the reaction system without sample; the A_{blank} is absorbance value of distilled water as blank group.

3.5. Superoxide radical scavenging assay

The superoxide radicals scavenging ability of COS and phenyl-acylchitooligosaccharide were assessed by the method of Zhong et al.

[20]. The capability of scavenging superoxide radical was calculated using the following equation:

$E\% = \left(1 - \frac{A_{sample}}{A_{control}} \right) \times 100$; among them, the A_{sample} is absorbance value of the experimental sample; the $A_{control}$ is absorbance value of the reaction system without sample.

3.6. Measurement of reducing power

The reducing power of COS and phenyl-acylchitooligosaccharide was assessed by the method of Zhong et al. [20]. The increase in absorbance indicates that the reduction ability is enhanced.

3.7. 1,1-Diphenyl-2-trinitrophenylhydrazine (DPPH) radical scavenging ability

The DPPH radical scavenging ability of COS and phenyl-acylchitooligosaccharide was assessed by the method of Curcio et al. [21]. The capability of scavenging DPPH radical was calculated using the following equation:

$E\% = 1 - \left[\left(\frac{A_1 - A_2}{A_0} \right) \times 100 \right]$; among them, the A_1 is absorbance value of the first measurement; the A_2 is absorbance value of the second measurement; the A_0 is absorbance value of the reaction system without sample.

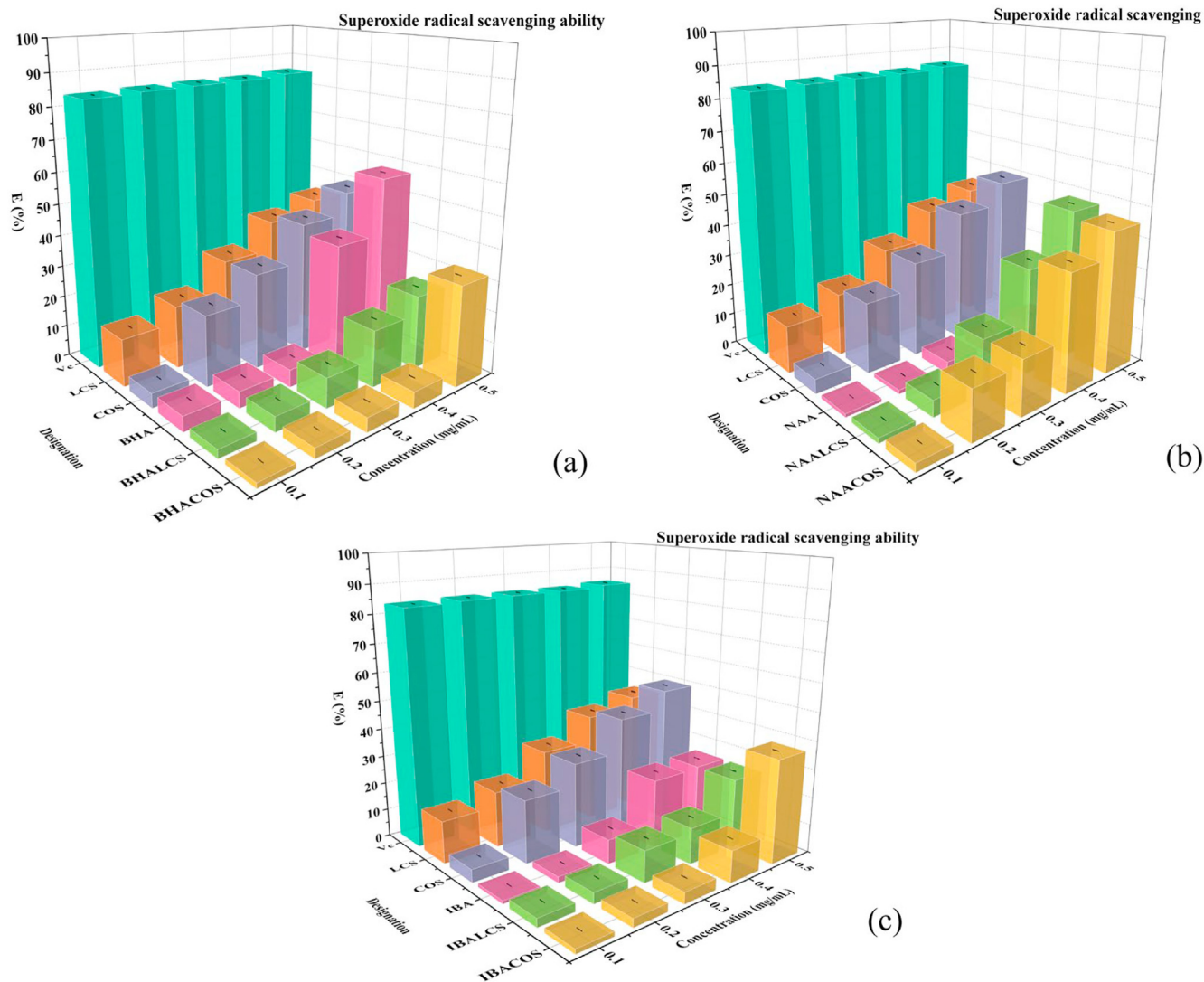


Figure 4. Scavenging effects of BHACS, NAACS and IBACS on superoxide radical. [(a) scavenging effects of BHACS, (b) scavenging effects of NAACS, (c) scavenging effects of IBACS]

3.8. Statistical analysis

All data are expressed as means \pm SD. Data were analyzed by an analysis of variance ($P < 0.05$) and the means separated by Dun-can's multiple range tests. The results were processed by computer programs: Excel, GraphPad Prism 5 and Origin 2021.

4. Results and discussion

4.1. Structure and physicochemical characteristics of the derivatives

Figure 1 presents the comparison of transmission FT-IR spectra data for phenyl-acylchitoooligosaccharide with original chitoooligosaccharide at different molecular weights. According to Figure 1, the characteristic broad peaks of stretching vibration of O–H and N–H located at $3500\text{--}3200\text{ cm}^{-1}$ are obviously shifted, indicating that N–H may react. The broad peak generated by the associated –OH stretching vibration is the most important feature of carboxylic acid, which is also the best proof for the acylation reaction between the target group and the chitoooligosaccharide. The characteristic absorption peak of –NH₂ at $1500\text{--}1510\text{ cm}^{-1}$ (namely the band of amide II) is enhanced, indicating that –NH₂ has undergone acylation reaction. The characteristic absorption peak of C=O stretching vibration (namely the amide I band) appears at $1640\text{--}1650\text{ cm}^{-1}$. And the C=C skeleton stretching vibration of aromatic

ring is also concentrated in this region. An obvious saturated –CH stretching vibration absorption peak appears at $2980\text{--}2940\text{ cm}^{-1}$. Located at $1210\text{--}1380\text{ cm}^{-1}$ are the absorption peaks of –CH₂ rocking vibration and asymmetric C2 deformation. The out-of-plane bending vibration of C–H on the aromatic ring is absorbed at $900\text{--}650\text{ cm}^{-1}$. The position of the absorption peak in this region has nothing to do with the nature of the substituents on the aromatic ring, but is related to the number of hydrogens connected to the aromatic ring. The lower the vibration frequency, the greater the absorption intensity. Located at 761 cm^{-1} is the characteristic absorption peak of ortho-disubstituted benzene ring. The characteristic absorption peaks of naphthalene ring are located at 780 cm^{-1} and 651 cm^{-1} . Located at 655 cm^{-1} is the characteristic absorption peak of indole ring. All the above results indicated that three kinds of phenyl-acylchitoooligosaccharide were obtained.

Figure 2 show the ¹³C NMR spectra of o-hydroxybenzoyl-chitoooligosaccharide (BHACS), α -naphthylacetyl-chitoooligosaccharide (NAACS), and 3-Indolebutyryl-chitoooligosaccharide (IBACS), respectively. According to the display of (a), at 174.61 ppm is the chemical shift of the C=O carbon of the amide bond. Located at 98.02(C1), 76.42(C4), 74.83(C5), 72.00(C3), 60.39(C6), 55.37(C2) ppm are the chemical shifts of the CS ring. Located at 116.43–160.53 ppm are the chemical shifts of the benzene ring skeleton of BHA. According to the display of (b), at 174.77 ppm is the chemical shift of the C=O carbon of the amide bond. Located at 98.05(C1), 76.43(C4), 74.84(C5), 72.11(C3), 61.83(C6), 55.38(C2) ppm

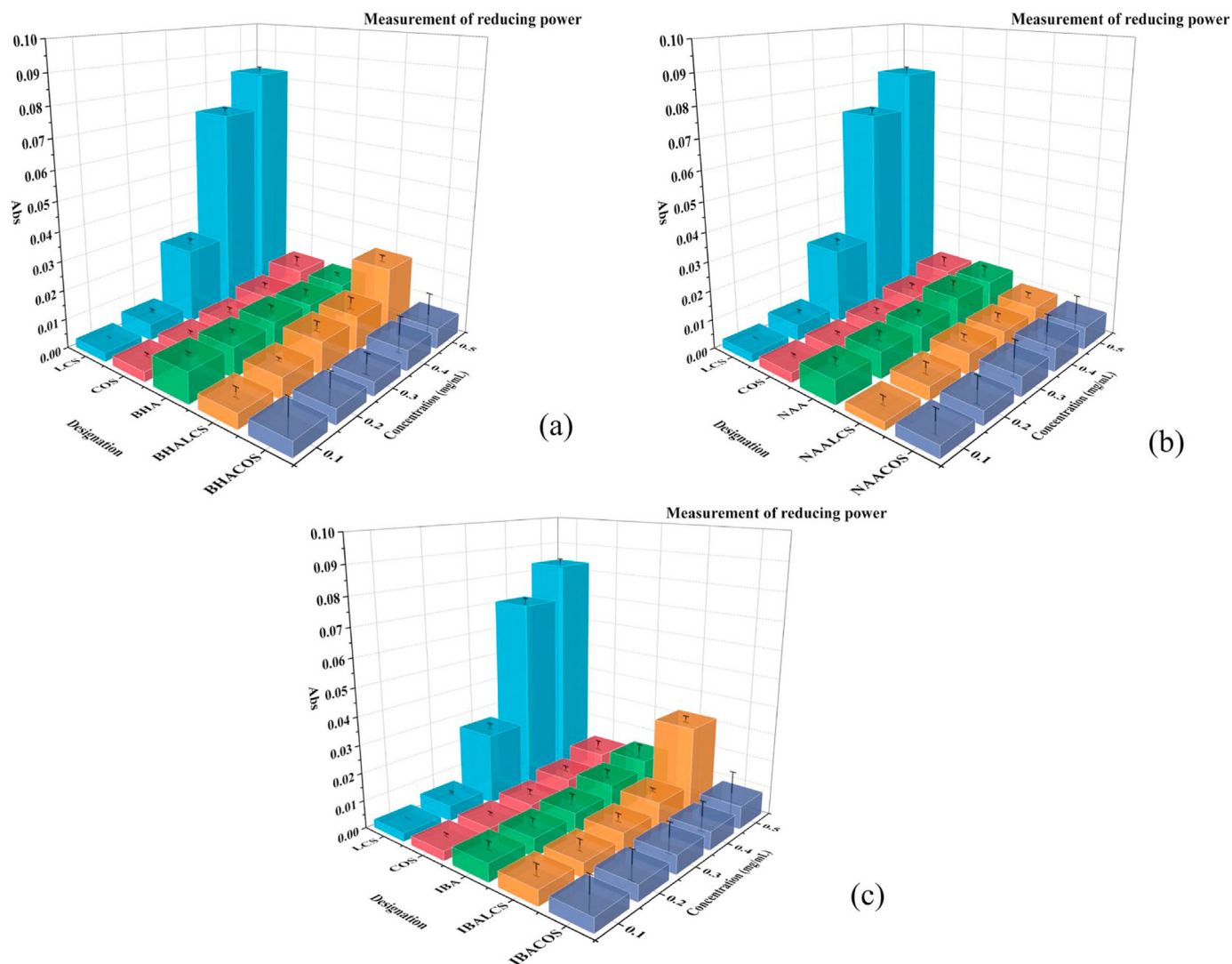


Figure 5. Reducing power of BHACS, NAACS and IBACS. [(a) reducing power of BHACS, (b) reducing power of NAACS, (c) reducing power of IBACS].

are the chemical shifts of the CS ring. Located at 133.61–160.61 ppm are the chemical shifts of the naphthalene ring skeleton of NAA. According to the display of (c), at 174.62 ppm is the chemical shift of the C=O carbon of the amide bond. Located at 98.07(C1), 76.44(C4), 74.26(C5), 72.04(C3), 60.40(C6), 55.38(C2) ppm are the chemical shifts of the CS ring. Located at 102.22–160.55 ppm are the chemical shifts of the indole ring skeleton of IBA. All the above results indicated that three kinds of phenyl-acylchitoooligosaccharide were obtained.

The results of elemental analyses, yield and grafted degree of the derivatives are listed in Table 1. The yields of BHACS, NAACS and IBACS were 46.84%, 41.11% and 49.18%, respectively. The elemental analyses indicate that the substitution degree of BHACS, NAACS, and IBACS about 74.47%, 28.62% and 71.12% respectively.

4.2. Hydroxyl radical scavenging activity of chitoooligosaccharide and phenyl-acylchitoooligosaccharide

Hydroxyl radicals are generated by the EDTANa₂-Fe (II)-H₂O₂ system. Safranin O can be uniquely targeted by hydroxyl radicals, causing it to fade. The content of hydroxyl radicals can be measured by colorimetry according to the degree of fading [22]. The scavenging ability of chitoooligosaccharide and phenyl-acylchitoooligosaccharide was determined based on this method. The results are plotted in Figure 3.

As shown in Figure 3, all derivatives have scavenging activity. The scavenging ability of the derivatives increased with increasing of the concentration. The highest clearance rates of derivatives in (a), (b), (c) were 11.43%, 8.29%, and 4.98%, respectively. Taking Vc as a reference, the IC₅₀ values of all derivatives were greater than Vc. The IC₅₀ value of BHALCS was 2.812 mg/mL, which was the best scavenging ability among the derivatives. For BHACS, NAACS and IBACS, the clearance rates of BHALCS, NAALCS and IBALCS were higher than those of BHACOS, NAACOS and IBACOS as a whole. This is because the high molecular weight derivatives have longer molecular chains, more connected groups, and more active hydrogens, which can react with more hydroxyl radicals to achieve the purpose of scavenging. However, because the low molecular weight derivatives have short molecular chains and also have many active hydrogens, the difference in the scavenging abilities of the two derivatives with different molecular weights is small. Moreover, the scavenging abilities of these three derivatives were lower than those of the three starting materials. This may be because the acylation reaction makes the amino group on the chitoooligosaccharide connect with the carboxyl group of the carboxylic acid, thus losing a lot of active hydrogen. Even the scavenging ability of the derivative is lower than that of the raw material itself. All the results showed that the scavenging ability of chitoooligosaccharide grafted containing benzene carboxylic acid was not significant, which needs further study.

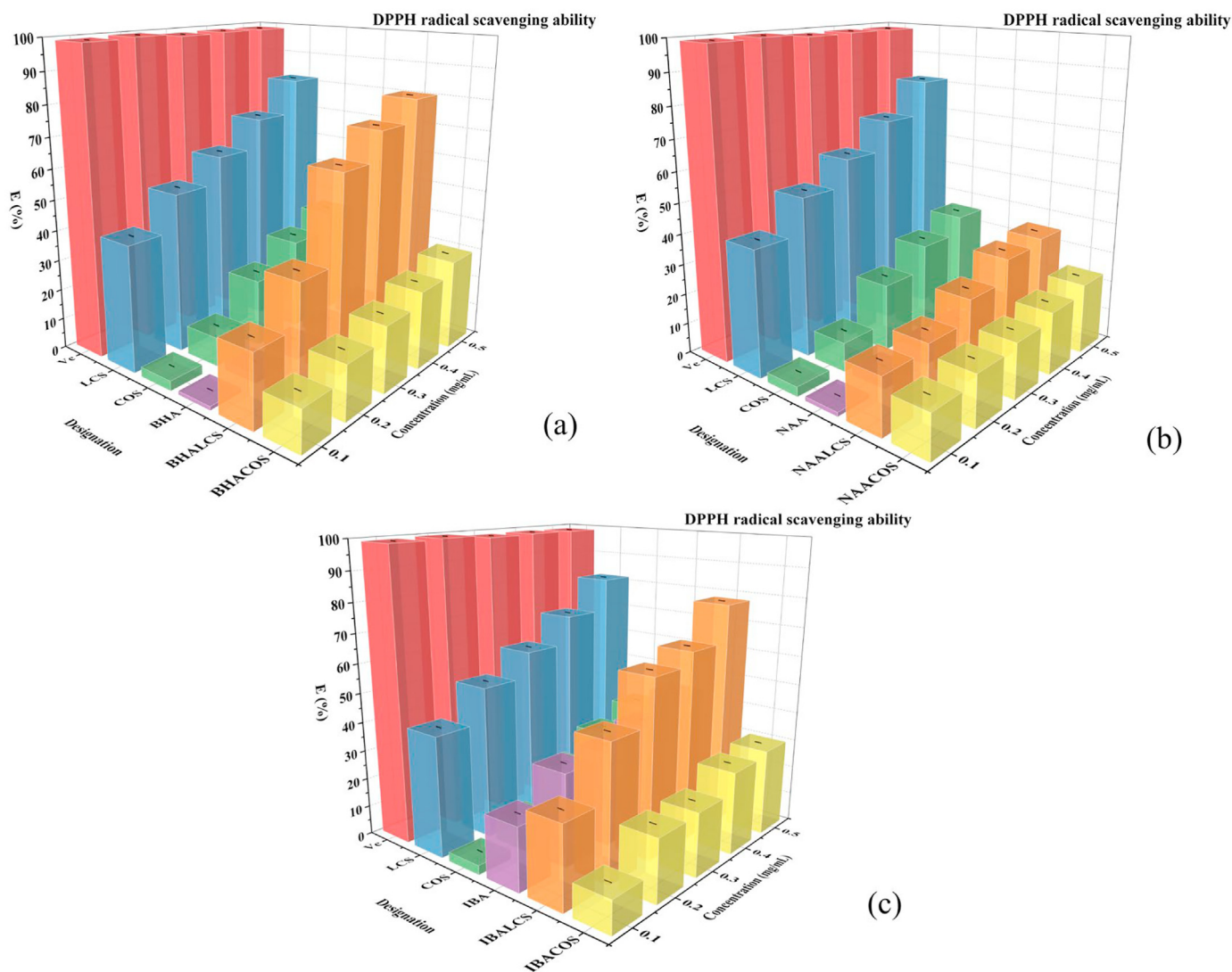


Figure 6. Scavenging effects of BHACS, NAACS and IBACS on DPPH radical. [(a) scavenging effects of BHACS, (b) scavenging effects of NAACS, (c) scavenging effects of IBACS]

4.3. Superoxide radical scavenging activity of chitoooligosaccharide and phenyl-acylchitoooligosaccharide

The main cause of oxygen toxicity in the body is due to the toxic effect of superoxide anion [23]. The results of superoxide anion scavenging with chitoooligosaccharide and phenyl-acylchitoooligosaccharide are shown in Figure 4.

The inhibitory effects of various phenyl-acylchitoooligosaccharide on superoxide anion free radicals were significant and enhanced with the increase of concentration. The highest clearance rates of derivatives in (a), (b), (c) were 32.13%, 47.44%, and 36.92%, respectively. For superoxide anion scavenging ability, both NAALCS and NAACOS were outstanding with IC50 values of 0.542 mg/mL and 0.546 mg/mL, respectively. The inhibitory ability of BHACOS, NAACOS and IBACOS was stronger than that of BHALCS, NAALCS and IBALCS. The removal of superoxide anion is mainly due to the hydrogen atom transfer mechanism, that is, the hydrogen atoms shed from the antioxidant are transferred to O_2^- . The number of free hydroxyl groups on the end groups of low molecular weight chitoooligosaccharides is higher than that of high molecular weight chitoooligosaccharides. And the introduction of BHA, NAA and IBA increases the number of active hydrogen atom. The hydrogen atoms on the O–H bond and N–H bond are shed and transferred to O_2^- , and the negative charge on O_2^- is transferred to the position where the hydrogen atoms are shed. To achieve free radical scavenging effect. However, the reason for the high scavenging ability of NAALCS and NAACOS is probably not due to the grafting of NAA. The degree of substitution of NAA is low, and most of the scavenging ability of its derivatives is derived from chitoooligosaccharide itself.

4.4. Reducing power of chitoooligosaccharide and phenyl-acylchitoooligosaccharide

The reducing power is usually associated with the presence of reductones, which act as antioxidants by donating a hydrogen atom to disrupt the free radical chain. Figure 5 show the reducing ability of various phenyl-acyl-chitoooligosaccharides.

The reducing ability of all derivatives showed a good linear relationship with concentration. The absorbance values in (a), (b), and (c) are all low, and the highest value is only 0.032 Abs. But the reducing ability of BHACS, NAACS and IBACS is very weak. The reducing power of no derivative can be higher than that of the chitosan oligosaccharide raw material. This is probably because the acylation reaction caused a large amount of damage to the reductone structure in the molecule, and then lost the reducing ability. Further research is needed on how to improve the reduction ability.

4.5. DPPH radical scavenging activity of chitoooligosaccharide and phenyl-acylchitoooligosaccharide

DPPH is 1,1-diphenyl-2-picrylhydrazino radical. There are multiple electron-withdrawing $-NO_2$ and benzene ring large π bonds in the molecule. It is an ideal pharmacological model for the detection of free radical scavenging activity [24]. Figure 6 show the scavenging ability of various phenyl-acylchitoooligosaccharide.

The scavenging ability of all derivatives is linear with concentration. The highest clearance rates of derivatives in (a), (b), (c) were 80.27%, 34.93%, and 77.66%, respectively. The BHALCS and IBALCS were significantly effective with IC50 values of 0.263 mg/mL and 0.253 mg/mL, respectively; these were lower than the IC50 values of BHA and IBA, 6.596 mg/mL and 0.663 mg/mL. The scavenging ability of BHACS and IBACS was higher than that of chitoooligosaccharide raw materials. The scavenging ability of NAACS is also limited by the low degree of substitution. The reaction system of the DPPH free radical scavenging experiment was anhydrous ethanol. The solubility of chitoooligosaccharide in absolute ethanol is very weak. However, the groups of derivatives (salicylic acid, α -naphthylacetic acid, indole-3-butyric acid)

have very high solubility in ethanol. This makes the scavenging effect of the derivatives on DPPH free radicals better than the above three tests. Some effects of different test system environments on the solubility and polarity of derivatives will indirectly interfere with the release of active hydrogen.

5. Conclusion

In this paper, six phenyl-acylchitoooligosaccharides were prepared. Their antioxidant activities were experimentally studied, and the following results were obtained: First, some derivatives have stronger superoxide anion free radical scavenging ability and DPPH free radical scavenging ability than chitosan oligosaccharide raw materials. Among them, the scavenging ability of NAALCS to superoxide anion was up to 47.44%, and the scavenging rate of BHALCS to DPPH free radical was up to 80.27%. Second, all the derivatives have lower scavenging ability to hydroxyl radicals than the reactants, and the reducing ability is also weak, the maximum value can only reach 0.032 Abs. The scavenging effect of hydroxyl radicals is also low, and the maximum value can only reach 11.43%. Third, different degrees of grafting in the derivatives have an effect on their antioxidant activity. Fourth, derivatives largely optimize the disadvantages of poor solubility of containing benzene carboxylic acids. All the results indicated that the phenyl-acylchitoooligosaccharide could improve partial antioxidant activity and water solubility. These experiments have important applications in both the pharmaceutical and plant nutrition fields. Salicylic acid, α -naphthylacetic acid and indole-3-butyric acid are all excellent phytostimulants, and the in vivo physiological activities of these three derivatives on plants will be studied in detail in later experiments. Their application prospects in the field of plant physiology are still broad. They also have certain application value in the aspects of food preservation and biological anti-oxidation and anti-aging.

Declarations

Author contribution statement

Yao Liu: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Fang Wen, Hehe Yang: Performed the experiments; Analyzed and interpreted the data.

Liangliang Bao: Analyzed and interpreted the data.

Zhihong Zhao: Contributed reagents, materials, analysis tools or data.

Zhimei Zhong: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Data availability statement

Data included in article/supplementary material/referenced in article.

Declaration of interest's statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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